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Seasonal patterns in immune indices reflect microbial loads on birds but not microbes in the wider environment

NICHOLAS P. C. HORROCKS,[1,]† KEVIN D. MATSON,[1] MOHAMMED SHOBRAK,[2] JOOST M. TINBERGEN,[1] AND B. IRENE TIELEMAN[1]

[1]*Animal Ecology Group, Centre for Ecological and Evolutionary Studies, University of Groningen, P.O. Box 11103, 9700 CC, Groningen, The Netherlands*
[2]*Biology Department, Science College, Taif University, P.O. Box 888, Taif 21974 Saudi Arabia*

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Abstract. Documenting patterns in immune function is a first step to understanding immune variation, but to comprehend causes and consequences, antigen and parasite exposure that may drive such variation must be determined. We measured host-independent microbial exposure in five species of larks (Alaudidae) in the Arabian Desert by sampling ambient air for culturable microbes during late spring and winter, two periods with contrasting environmental conditions. We developed a novel technique to assay densities of microbes shed from birds, and we quantified four indices of constitutive innate immunity. Birds shed significantly more microbes during spring than winter, and all immune indices except one were also significantly higher during spring. In contrast, concentrations of airborne environmental microbes were higher in winter. Among all birds in both seasons, lysis titers were positively correlated with total densities of microbes shed from birds, suggesting that immune defenses are directed towards the microbes that birds carry, rather than microbes in the wider environment. Our findings highlight the relevance of quantifying non-specific immune challenges in ecological immunology studies, and reinforce the importance of both host-dependent and host-independent measures of antigenic pressure for understanding immune variation.

Key words: air sampling; *Alaudidae*; constitutive innate immunity; desert; ecological immunology; lark; microbial pressure.

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† **E-mail:** n.p.c.horrocks@rug.nl

INTRODUCTION

Explanations for immunological variation originate from two different, but related, viewpoints. Following life-history theory (Roff 1992, Stearns 1992), the immune system competes for limited resources with other physiological and ecological processes (Sheldon and Verhulst 1996, Norris and Evans 2000, Schmid-Hempel 2003). Investment in resource-demanding activities such as reproduction (Ilmonen et al. 2000) or migration (Buehler et al. 2010) might result in decreased investment in immune function. By shifting focus to infections and other threats to fitness, immune system variation can instead be examined through its benefits rather than its costs. Since parasites and pathogens reduce host fitness (Brown et al. 1995, Fitze et al. 2004), evolutionary theory suggests that investment in defenses should be higher when the negative consequenc-

es of parasites and pathogens are greater (Tschirren and Richner 2006). Differences in disease threats that are a function of environment, time, or other ecological factors (e.g., Piersma 1997, Guernier et al. 2004) might therefore lead to parallel differences in immune function.

These two perspectives and the processes underlying them are not mutually exclusive. For example, seasonality, one type of environmental variation, could drive immune variation through either or both of the processes described above. Seasonal variation in immunity might arise if resource trade-offs change over the annual cycle (Buehler et al. 2008, Martin et al. 2008), for example when thermoregulatory demands or food availability vary (Nelson et al. 2002). Alternatively, seasonal variation in immune defense may occur if disease risk changes over the annual cycle (Nelson et al. 2002). Seasonal differences in the abundance of disease-transmitting vectors (Franklin and Whelan 2009) or in the frequency of interactions between conspecifics might alter the risk of parasite transmission (Sheldon 1993), injury or infection (Zuk and Johnsen 1998). Variation in weather may affect microbial exposure: heat and humidity promote contact with fungal spores (Talley et al. 2002); wind increases encounters with airborne microbes (Jones and Harrison 2004).

To understand how changes in disease risk shape immune variation over the annual cycle requires measuring host-dependent and host-independent parasites, pathogens and microbial assemblages (Horrocks et al. 2011*a*). Microbial assemblages, in particular, can offer an informative and expansive view into the antigenic pressures faced by wild animals. Microbes are found in essentially all environments and can be encountered by all individuals, since co-evolved vectors are not required for their transmission. Higher concentrations of airborne microbes lead to increased inflammation and more dead macrophages in vitro (Huttunen et al. 2010). Host-associated microbial assemblages of wild animals may show connections to local environmental characteristics (Klomp et al. 2008) and relate directly to immunological investment (Alcaide et al. 2010).

We developed and implemented a novel air-sampling method to measure concentrations of culturable microbes in ambient air and densities of microbes shed from the surface of birds. This dual approach advances ecological immunology and builds upon previous studies by assessing components of disease pressure (i.e., microbial abundances) that are strictly independent of host defenses. Such host defenses include constitutive innate immunity, which may be particularly relevant for defense against the continual antigenic pressure posed by airborne and bird-associated microbes because it is always active, and is not specific to particular pathogens (Janeway et al. 2004). Important elements of constitutive innate immunity include the acute phase proteins, natural antibodies, and lytic enzymes (Janeway et al. 2004). Acute phase proteins, such as haptoglobin and ovotransferrin, increase in concentration during inflammation or infection (van de Crommenacker et al. 2010, Horrocks et al. 2011*b*), and limit microbial growth (Cray et al. 2009). Natural antibodies recognize foreign particles such as invading microorganisms and mark them for phagocytosis. These non-specific antibodies also activate the complement system, an enzyme cascade that leads to cell lysis (Ochsenbein and Zinkernagel 2000).

The aims of this study were to assess seasonal differences in microbial abundance in ambient air and on birds, and to investigate how these differences related to immunity. We studied three resident and two nomadic lark species (Alaudidae) in the Arabian Desert, a well-studied system (reviewed in Tieleman 2005), where environmental conditions differ markedly between seasons. We compared birds during late spring, when they breed, and in winter, when birds are reproductively inactive. During late spring high levels of solar radiation and temperatures may severely limit microbial growth and survival (Tong and Lighthart 1997, Talley et al. 2002, Burrows et al. 2009, Tang 2009). Simultaneously, high temperatures may affect immune defenses through resource trade-offs: restricted food availability and activity budgets negatively impact activities such as preening (Tieleman and Williams 2002) that might otherwise reduce microbial loads, while reduced basal metabolic rates (Tieleman et al. 2003*b*) might also have repercussions for immune functioning (Tieleman et al. 2005). In contrast, lower temperatures in winter may relax time and food constraints, and allow

Table 1. Weather conditions at Mahazat as-Sayd, Saudi Arabia, based on historic data (National Wildlife Research Center, *unpublished data*). Data are for spring (May and June) and winter (November and December) and represent the monthly mean values (± SD). In the case of relative humidity and solar radiation the mean of the absolute maximum values are given. For temperature, the maximum values are given in parentheses.

Season	Rainfall (mm)	Temperature (°C)	Relative humidity (%)	Solar radiation (W/m^2)
Spring	4.1 ± 6.1	29.3 (36.9)	44.9 ± 17.4	896.3 ± 139.4
Winter	11.5 ± 18.3	18.4 (24.2)	70.2 ± 19.3	692.3 ± 109.9

for enhanced self-maintenance, but microbial pressures could increase. Thus, immune indices could match patterns in airborne and bird-associated microbes, or could reflect broader seasonal differences in avian physiology. To explore the potential impact of environmental variation on immune function further, we compared resident larks with nomadic larks, which ostensibly move location to keep their ecological setting more constant, and we analyzed inter-annual variation in immune defenses between consecutive springs.

Methods

Study species and study locations

We sampled larks in late spring 2006 (May 11–June 12), late spring 2007 (May 4–June 6) and in winter 2007 (November 24–December 16). In spring 2006 and winter 2007 hoopoe larks (*Alaemon alaudipes*), Dunn's larks (*Eremalauda dunni*), bar-tailed desert larks (*Ammomanes cincture*), black-crowned finchlarks (*Eremopterix nigriceps*) and crested larks (*Galerida cristata*) were captured at Mahazat as-Sayd ('Mahazat'), a reserve in central Saudi Arabia (22°15′ N, 41°50′ E). Mahazat is characterized by sparsely-vegetated gravel plains. Annual mean rainfall is 90 ± 76 mm (± SD). Spring conditions are hot and dry (Table 1; Tieleman and Williams 2002). If there has been sufficient rainfall in the preceding months, then some green vegetation is available, providing invertebrate and plant food for birds. In winter, temperatures are lower and rainfall is higher. If environmental conditions are extreme in spring and summer, then the two nomadic species, black-crowned finchlark and crested lark, leave Mahazat (B. I. Tieleman, *personal observation*). This was the case in spring 2007, when we caught both species ~170 km away at the National Wildlife Research Center, Taif (21°15′ N, 40°42′ E) where conditions are more benign (Tieleman et al. 2003*a*). We trapped birds with mist nets or clap traps. Permission was granted by the National Wildlife Research Center, Saudi Arabia.

Environmental and bird sampling

We sampled the environment (2007 only; see 'Environmental air-sampling') and birds (2006 and 2007) independently of each other. In spring and winter 2007, immediately upon capture, birds ($n = 77$) were air-sampled to measure microbial densities (see 'Bird air-sampling') and then bled (45–60 minutes after capture), weighed (±0.1 g) and measured (wing, ±0.1 cm; tarsus, ±0.01 cm). A further 67 birds in 2007, and all birds in spring 2006 ($n = 62$), were only bled (<10 minutes after capture), weighed and measured. Blood was collected from the brachial vein (200–300 μl) and stored on ice for 30 minutes to four hours until it was centrifuged to separate plasma and cellular fractions. Plasma was frozen and stored at −20°C until use in immune assays. Birds were sexed by body measurements and by behavioral observations.

Air sampling

We used a portable impaction air sampler for agar plates (Burkard, Rickmansworth, UK), which draws air at a constant rate over a metal 'sieve plate' perforated with 1.0 mm holes. Under the sieve plate, an agar-filled Petri dish collects microbial particles passing through the holes (Fig. 1A). The number of colony-forming units (CFUs; Fig. 1B) that grow during incubation of the plate provides an index of the concentration of culturable airborne microbial particles. Culture-based data from air-sampling devices are useful indexes of overall microbial concentrations

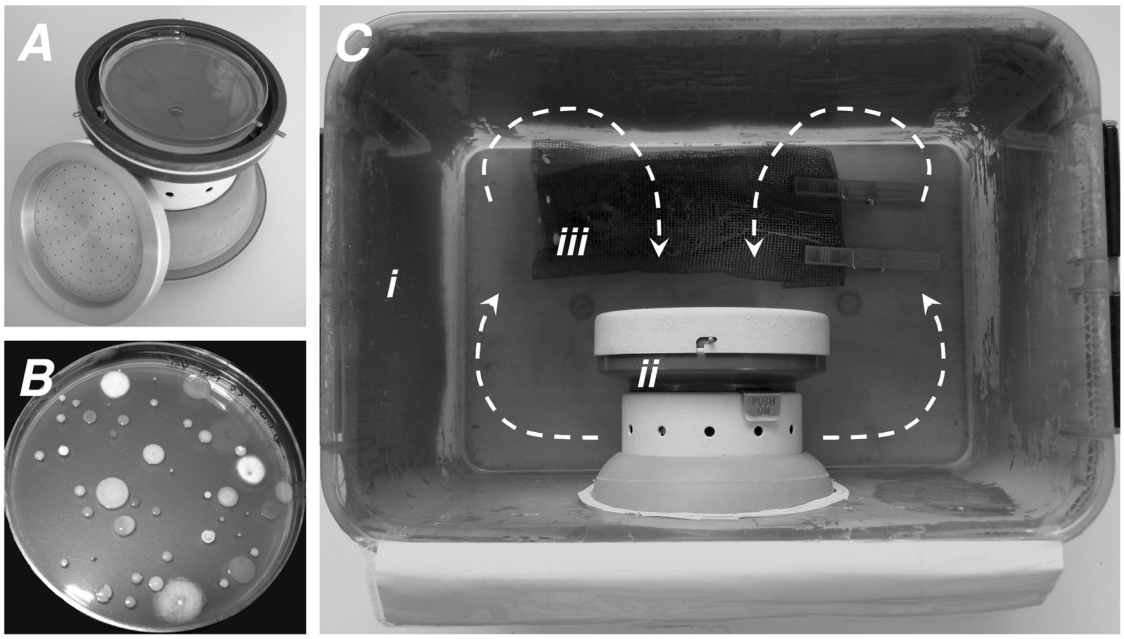

Fig. 1. Air sampler and set-up for air-sampling of birds. (A) Air sampler with sieve plate removed to show the agar plate in place. (B) An agar plate after incubation. (C) The bird air-sampling experimental set-up: (i) pre-sterilized, darkened plastic box; (ii) air sampler; (iii) bird restrained in soft mesh sterilized tube. Air passes over the bird and onto the agar plate (top arrows) and is then recycled back to the box through the air vents of the sampler (bottom arrows).

(e.g., Haas et al. 2010), since they provide an easily understandable measure of antigenic exposure, despite issues pertaining to culturability (Rappé and Giovannoni 2003).

We collected two types of data using the air sampler: concentrations of culturable microbes in ambient air and densities of culturable microbes shed by birds. In both cases we used three agars to culture different microbial assemblages: (1) generalist aerobic bacteria (Tryptic soy agar; Sigma-Aldrich, St Louis, MO, USA); (2) gram-negative bacteria (MacConkey agar with crystal violet, sodium chloride, and 0.15% bile salts; Sigma-Aldrich), and (3) fungi (Sabouraud 4% Glucose agar (Sigma-Aldrich), with 50 mg/L Gentamicin antibiotic (Invitrogen, Breda, The Netherlands)). Plates were incubated at 30°C, but colonies grew at different rates, so numbers of CFUs were counted after 24 hours (generalist bacteria), 72 hours (fungi) and 96 hours (gram-negative bacteria). We applied a correction, calculated for a 100-hole air sampler (Andersen 1958), to all CFU counts. This correction adjusts for the possibility that multiple culturable particles pass through a sampling hole in the sieve plate and produce only one countable colony.

Environmental air-sampling

Environmental air samples were collected at different times of the day to account for potential diurnal variation (Tong and Lighthart 1999), and over a series of days (spring $n = 20$ days, winter $n = 16$ days) that spanned each fieldwork period. Sampling sites were chosen to represent the range of micro-habitats that birds might encounter, but environmental air samples do not correspond with specific bird air-sampling events. We identified sampling sites by noting where birds foraged and rested on the ground as well as areas they crossed in order to reach different foraging patches. Birds did not differ in their use of microhabitats between seasons, so the same sites ($n = 10$) were sampled at Mahazat during both spring and winter in 2007. Five sites at Taif were sampled only in spring 2007, when we also caught birds there. During spring, sites were sampled in the order they were identified; sites were sampled in the same order during

winter. Environmental air was sampled for 15–30 minutes per agar plate. We standardized sampling effort by multiplying the number of minutes by the air flow rate (20 L/min) and expressed data as concentrations (CFU/m^3 of air).

Bird air-sampling

We sampled bird-associated microbes by isolating a bird in a sterile box and using the air sampler to collect the microbial particles shed from the bird as air passed over it. Birds were air-sampled immediately after capture. Handling was kept to a minimum, and before touching a bird we cleaned our hands with antibacterial hand wash. Single-use paper bags were used as handling bags to avoid cross-contamination among birds. Birds were restrained inside a soft mesh tube that was sterilized with ethanol and placed, always oriented the same way relative to the air sampler, inside a sterilized plastic box (33 $\times$ 22 $\times$ 16 cm) with fitted lid. The sterilized head of the air sampler was fitted through a hole in the side of the box so that it circulated air back into the box during sampling (Fig. 1C). The order of the three agar types varied among birds, but duplicate plates were run for each agar. Birds were always sampled for five minutes per plate. Since species differed in body size, we determined body surface area (Walsberg and King 1978) and expressed counts as densities (CFU/cm^2 of body surface area). Because a standard sampling time was always used and since air in the sampling chamber was recycled, these data are not based on air volume.

Immune assays

We determined haptoglobin concentrations (mg/ml) using a functional assay that measures the haem-binding capacity of plasma (TP801; Tri-Delta Diagnostics, NJ, USA; 'manual method'). Ovotransferrin concentrations (mg/ml) were measured according to Horrocks et al. (2011*b*) with the exception of bar-tailed desert larks and black-crowned finchlarks, for which plasma volumes were insufficient. We measured natural antibody-mediated agglutination titers and complement-mediated lysis titers using rabbit red blood cells (B-0009D, Harlan, UK), using the assay of Matson et al. (2005).

Statistical analyses

We analyzed concentrations of airborne microbial particles with Mann-Whitney U tests because data were not normally distributed. We analyzed bird-associated microbial densities with generalized linear models with Poisson (or quasi-Poisson in cases of over-dispersion) errors. Full models initially contained the fixed terms species, season, sex, and the interactions species $\times$ season and species $\times$ sex. We did not include interactions involving season and sex because for some species we only captured one sex during a season. With one exception (see Results section, 'Inter-annual variation in immune indices'), re-running analyses for each sex separately did not change the main findings and we do not report these results. We included time of day as a covariate because concentrations of airborne microbes can fluctuate diurnally (Tong and Lighthart 1999).

Before examining seasonal and inter-annual differences in immune indices we explored the effects on immunity of time delay between capture and blood collection. This differed between air-sampled (45–60 minutes) and non-air-sampled birds (<10 minutes) but had no effect on immune indices (all $P > 0.1$), so we combined data in further analyses. We also explored the effects of nomadic status and location (spring 2006 and winter 2007: residents and nomads at Mahazat; spring 2007: residents at Mahazat, nomads at Taif) on immune indices and bird-associated microbes. The interaction nomadic status $\times$ season (all $P > 0.08$) and the term nomadic status (all $P > 0.3$) were non-significant for all measures of bird-associated microbes and immunity. Comparing immune indices of birds sampled in spring 2006 and spring 2007, the interaction nomadic status $\times$ year (all $P > 0.2$) and the term nomadic status (all $P > 0.3$) were also non-significant. Therefore we combined data from Mahazat and Taif and from resident and nomadic species in analyses of seasonal and inter-annual variation. We then used analysis of variance to examine the effects of season or year for each immune variable. Full models contained the fixed terms season or year, species, sex, species $\times$ sex and, when relevant, species $\times$ season.

We examined correlations between total microbial density (the sum of generalist, gram-

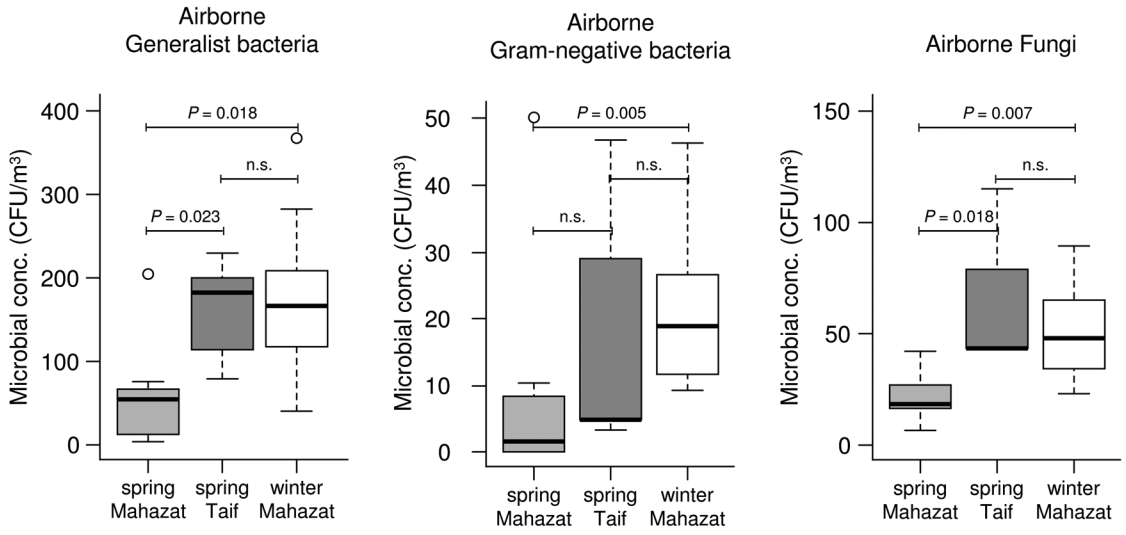

Fig. 2. Concentrations of airborne microbes (generalist and gram-negative bacteria, and fungi) measured in the Arabian Desert at Mahazat as-Sayd and Taif in spring and at Mahazat as-Sayd in winter. Boxes encompass all data between the 25th and 75th percentiles (interquartile range, IQR). Thick bars inside boxes indicate the median value. 'Whiskers' indicate either the minimum or maximum value, or 1.5 times the IQR (approximately two standard deviations), whichever is smaller. Data outside this range ('outliers') are plotted individually as open circles.

negative and fungal densities per bird) and each immune index with generalized linear mixed models. We included species means of total microbial density and individual deviations from these means as explanatory variables, to distinguish between within- and among-species variation (van de Pol and Wright 2009). Additionally, initial models contained the fixed effects season, the random effect species, and the interactions species-mean × season and individual deviation × season.

Terms were dropped sequentially when $P > 0.05$. We report interaction terms only when significant. Differences among levels were examined using post-hoc Tukey tests. Residuals of statistical models were examined graphically for normality and homogeneity of variance and met these assumptions. Analyses were performed using R 2.10.1 (R Development Core Team 2009).

Results

Seasonal patterns in environmental airborne microbes

Concentrations of culturable airborne microbes at Mahazat were significantly higher in winter than in spring for all three groups of microbes (Figs. 2 and 3). In both seasons, concentrations of generalist bacteria were highest and gram-negative bacteria were lowest. Spring concentrations of airborne microbes at Taif were higher than spring concentrations at Mahazat and were not significantly different from winter concentrations at Mahazat (Fig. 2).

Seasonal patterns in bird-associated microbes

Birds shed significantly higher densities of generalist and gram-negative bacteria in spring than in winter (generalist bacteria, $F_{1,71} = 7.90$, $P = 0.006$; gram-negative bacteria, $F_{1,72} = 4.78$, $P = 0.032$; Figs. 3 and 4), when accounting for differences among species. Densities of fungal CFUs sampled from birds did not differ significantly between the two seasons ($F_{1,62} = 2.92$, $P = 0.093$) but followed a similar trend (Figs. 3 and 4). In both seasons, densities of generalist bacteria were highest, and densities of gram-negative bacteria were lowest, matching patterns exhibited in ambient air. Densities of microbes shed by males and females did not differ (all $P > 0.2$), and for generalist and gram-negative bacteria there was no effect of the time of day when

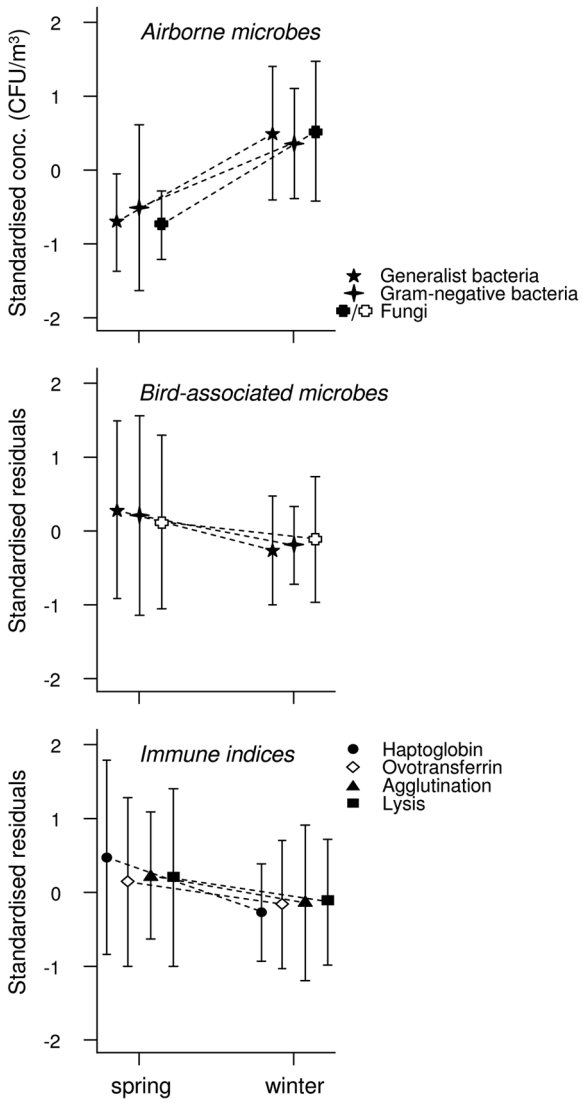

Fig. 3. Differences between spring and winter in concentrations of airborne microbes, densities of bird-associated microbes and indices of constitutive innate immunity measured in five lark species in the Arabian Desert. Data are mean values and error bars indicate standard deviations. In the lower two panels data are plotted as standard residuals extracted from the final statistical model for each variable. Non-significant differences between seasons for a particular variable are represented by open symbols.

birds were sampled ($P > 0.1$). Densities of fungal CFUs increased over the course of the day ($F_{1,62} = 5.98$, $P = 0.017$) although the size of this effect was very small (an increase of less than two CFU/cm^2 per 12 hours). When accounting for the effect of season, significant differences existed among species for densities of generalist bacteria ($F_{4,71} = 2.93$, $P = 0.027$) and gram-negative bacteria ($F_{4,72} = 2.99$, $P = 0.024$) but not for densities of fungi ($F_{4,62} = 1.88$, $P = 0.126$). A post-hoc test revealed that crested larks shed significantly more generalist bacteria than the four other species, which otherwise did not differ (Fig. 4). A post-hoc test could not resolve any significant differences among species for densities of gram-negative bacteria, despite the significance of species in the main model.

Seasonal patterns in immune indices

Haptoglobin concentrations ($F_{1,133} = 19.21$, $P < 0.001$) and agglutination ($F_{1,133} = 4.24$, $P = 0.041$) and lysis ($F_{1,129} = 5.10$, $P = 0.026$) titers were significantly higher in spring than in winter (Figs. 3 and 5). Ovotransferrin concentrations showed a similar but non-significant trend ($F_{1,39} = 0.92$, $P = 0.343$; Figs. 3 and 5). Males had significantly higher haptoglobin concentrations than females ($F_{1,133} = 5.33$, $P = 0.023$). Other immune indices did not differ between sexes. Lysis was the only immune parameter where species differed significantly ($F_{4,129} = 3.37$, $P = 0.012$; Fig. 5). A post-hoc test revealed the significance of this term was driven by a single species pair (Dunn's lark > bar-tailed desert lark).

Inter-annual variation in immune indices

Haptoglobin concentrations ($F_{1,108} = 9.05$, $P = 0.003$) and agglutination ($F_{1,93} = 6.40$, $P = 0.013$) and lysis ($F_{1,95} = 31.16$, $P < 0.001$) titers were significantly higher in spring 2007 than in spring 2006. Ovotransferrin concentrations showed a similar trend but were not significantly different between years ($F_{1,41} = 3.27$, $P = 0.078$). When analyzing the sexes separately (see Statistics section), for females only there was a significant species × year interaction in agglutination titers ($F_{3,22} = 4.48$, $P = 0.013$). Females of resident species (Hoopoe Lark and Dunn's Lark) exhibited higher agglutination titers in spring 2007 compared to spring 2006, while the reverse was true for the two nomadic species (black-crowned finchlark and crested lark). Relative to the mean, annual variation in all immune indices was greater than seasonal variation in the same indices (Table 2).

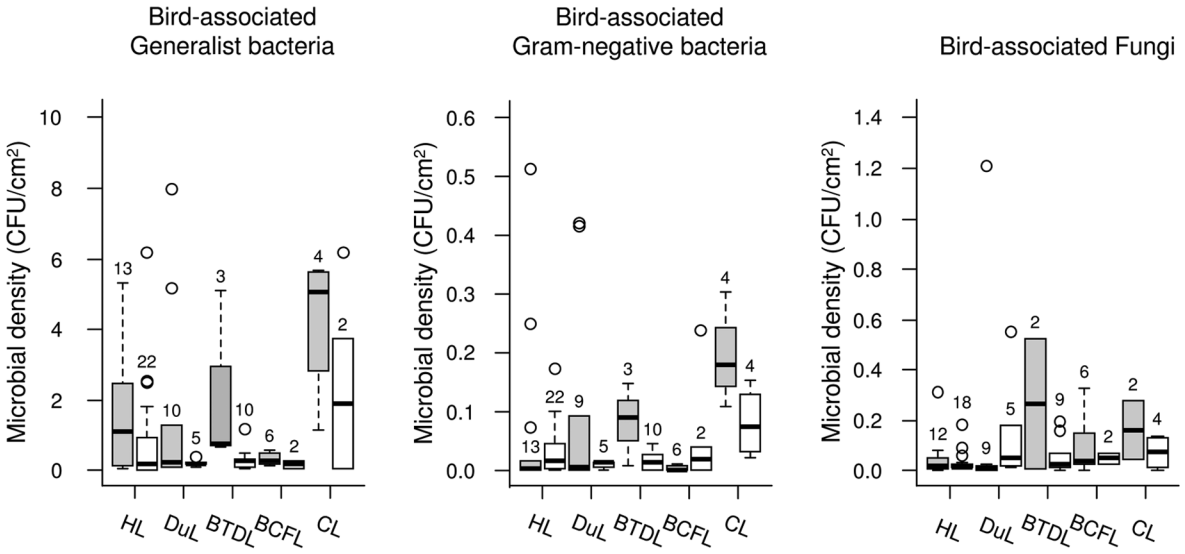

Fig. 4. Densities of bird-associated microbes (generalist and gram-negative bacteria, and fungi) air-sampled from five species of lark in the Arabian Desert in spring (grey bars) and winter 2007. Numbers above bars are sample size. HL = hoopoe lark; DuL = Dunn's lark; BTDL = bar-tailed desert lark; BCFL = black-crowned finchlark; CL = crested lark. Boxes encompass all data between the 25th and 75th percentiles (interquartile range, IQR). Thick bars inside boxes indicate the median value. 'Whiskers' indicate either the minimum or maximum value, or 1.5 times the IQR (approximately two standard deviations), whichever is smaller. Data outside this range ('outliers') are plotted individually as open circles.

Correlations between total microbial density and immune indices

Lysis titers correlated positively and significantly with total densities of microbes shed by birds ($F_{1,65} = 4.09$, $P = 0.047$; Fig. 6), after controlling for season ($F_{1,65} = 1.35$, $P = 0.249$) and among-species variation ($F_{1,3} = 0.57$, $P = 0.506$). Haptoglobin and ovotransferrin concentrations and agglutination titers showed no significant relationships with total microbial load, either at the among-species level (all $P > 0.4$) or at the within-species level (all $P > 0.1$).

Discussion

We found strong seasonal patterns in both immune indices and environmental and bird-associated microbial assemblages. Densities of bird-associated microbes and immune indices were higher in spring and lower in winter, while the reverse pattern was observed for concentrations of airborne microbes (Fig. 3). Therefore, microbial loads carried by birds might shape immunity more than concentrations of microbes in the wider environment. This conclusion is strengthened by observations of the two nomadic species, crested lark and black-crowned finchlark: in 2007 these species encountered similar concentrations of airborne microbes at Taif during the spring and at Mahazat during the winter (Fig. 2), but exhibited different densities of bird-associated microbes (Fig. 4) and immune defenses between seasons (Fig. 5). We conclude that explanations for seasonal variation in immune function should incorporate antigenic pressure and not be restricted to resource-allocation trade-offs between immunity and other physiological functions (Horrocks et al. 2011*a*). Quantifying both host-dependent and host-independent microbial loads to map 'threat landscapes' is possible with new techniques, including the novel air-sampling approach used in this study.

Airborne and bird-associated microbes followed opposite spring-winter patterns

Levels of airborne microbes were consistently and significantly higher in winter, with concen-

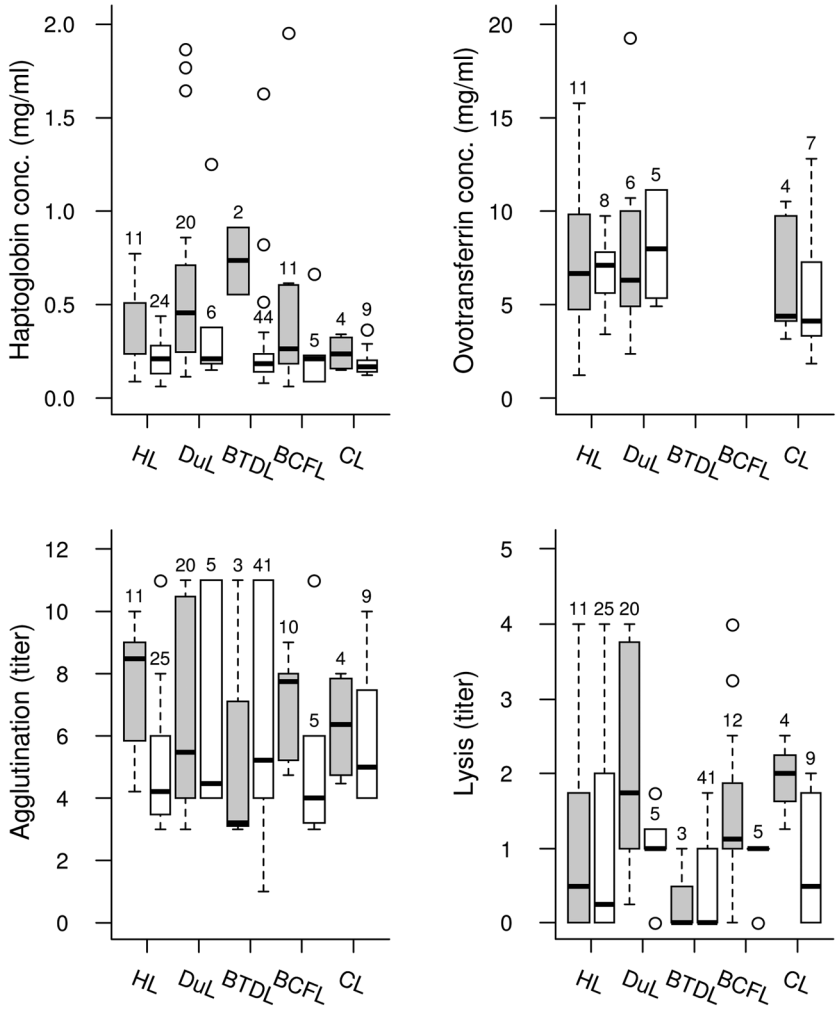

Fig. 5. Haptoglobin and ovotransferrin concentrations, and agglutination and lysis titers measured in five species of lark in the Arabian Desert in spring (grey bars) and winter 2007. Numbers above bars are sample sizes. HL = hoopoe lark; DuL = Dunn's lark; BTDL = bar-tailed desert lark; BCFL = black-crowned finchlark; CL = crested lark. Ovotransferrin concentrations are missing for BTDL and BCFL because insufficient sample volumes were available to measure this protein in these species. Boxes encompass all data between the 25th and 75th percentiles (interquartile range, IQR). Thick bars inside boxes indicate the median value. 'Whiskers' indicate either the minimum or maximum value, or 1.5 times the IQR (approximately two standard deviations), whichever is smaller. Data outside this range ('outliers') are plotted individually as open circles.

trations of gram-negative bacteria and fungi at least double those measured in spring (Fig. 3). Previously-reported seasonal patterns of concentrations of airborne microbes match the patterns we found for fungi (Abdel-Hafez 1984, Al-Suwaine et al. 1999*a*, Al-Suwaine et al. 1999*b*), but not for bacteria (Burrows et al. 2009). However, only a single study reports seasonal patterns of bacterial concentrations in a desert environment, with inconsistent patterns: bacterial concentrations are higher in spring at half of the sampling sites, but higher in winter or not seasonally variable at the remainder (Mahdy and El-Sehrawi 1997). Perhaps counter to other non-desert locations, conditions for microbial growth at Mahazat might actually be more favorable in winter than spring, due to reductions in temperature and solar radiation, and increases in rainfall

Table 2. Mean values (intercept), estimate sizes (difference) and 95% confidence intervals (95% CI) around these estimates for four indices of constitutive innate immunity and body mass from five species of larks in the Arabian Desert in spring and winter 2006 and spring 2007. The intercepts and estimates are taken from final statistical models examining the role of (a) seasonal and (b) annual effects on each parameter. The intercept indicates the mean value of the reference category ('spring' for seasonal variation, '2006' for annual variation) and estimates are given for the other category ('winter' and '2007') relative to this.

Source of variance	Parameter	Mean (intercept)	Difference	95% CI
(a) Seasonal				
Spring vs. winter	haptoglobin (mg/ml)	0.42	−0.25	−0.36 to −0.14
	ovotransferrin (mg/ml)	10.15	−2.29	−7.12 to 2.54
	agglutination (titer)	6.99	−1.10	−2.16 to −0.04
	lysis (titer)	1.46	−0.52	−0.98 to −0.06
	mass (g)	15.10	−1.35	−2.44 to −0.26
(b) Inter-annual				
2006 vs. 2007	haptoglobin (mg/ml)	0.30	0.19	0.07 to 0.32
	ovotransferrin (mg/ml)	6.35	3.59	−0.42 to 7.60
	agglutination (titer)	5.86	1.13	0.24 to 2.02
	lysis (titer)	0.37	1.27	0.82 to 1.73
	mass (g)	13.46	1.67	0.48 to 2.85

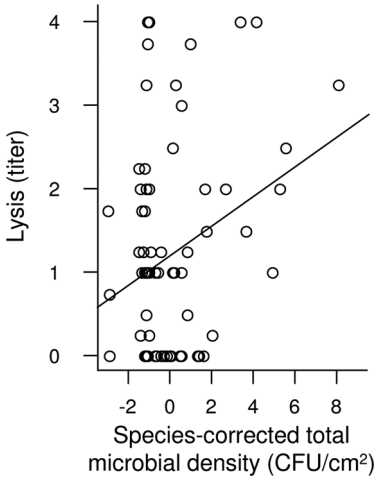

Fig. 6. The relationship between lysis titer and total microbial load, pooling all individuals from five species of larks from the Arabian Desert.

and relative humidity (Tong and Lighthart 1997, Talley et al. 2002, Burrows et al. 2009, Tang 2009).

Given the strong seasonal pattern in concentrations of airborne microbes, it is particularly intriguing to find such a clear pattern in the opposite direction for the densities of microbes shed by birds (Fig. 3). Birds in our study population molt in summer. Thus, feathers of spring-sampled birds were older than those of winter-sampled birds. Older feathers may harbor more microbes, due to increased time for colonization to occur (Burtt and Ichida 1999, Bisson et al. 2007, although see Giraudeau et al. 2010). Additionally, birds in spring might put less effort into feather maintenance if preening carries less value for old feathers, if they are harder to clean, or if time-budgets are restricted (Tieleman et al. 2003*c*, Lucas et al. 2005, Tieleman et al. 2008). High spring temperatures restrict preening time in hoopoe larks, which spend only 2.4% of the day preening (Tieleman and Williams 2002), compared to an average of 9.4% for other bird species (Cotgreave and Clayton 1994). Other behavioral differences between spring and winter may also affect bird-associated microbial densities. To escape the midday heat during spring and summer, desert-living larks lay down in shallow scrapes in shade-spots or in lizard burrows to conduct away heat (Williams et al. 1999). Such behavior might increase exposure to dust and soil microbes (Shawkey et al. 2005, but see Bisson et al. 2007).

Despite opposing seasonal patterns, the relative abundance of the three microbial groups was the same in the air, and on birds (generalist bacteria > fungi > gram-negative bacteria) and also did not change between seasons. Thus, factors shaping relative abundance of these microbial groups in the wider aerial environment (e.g., temperature, humidity) might also be

important for shaping microbial assemblages on the plumage of birds (Bisson et al. 2007). Interspecific variation in densities of bird-associated microbes might arise from differences in exposure or in feather characteristics such as preen-wax composition (Shawkey et al. 2003, Reneerkens et al. 2008). Crested larks had the highest densities of generalist bacteria and gram-negative bacteria (Fig. 4). The most cosmopolitan of the five study species, crested larks occupy the widest variety of habitats (del Hoyo et al. 2004), including areas of human habitation, where concentrations of airborne microbes are hypothesized to be higher than in natural surroundings (Burrows et al. 2009). Therefore, the higher densities of microbes on crested larks may reflect greater exposure.

Seasonal variation in constitutive innate immunity

Immune indices were consistently higher in spring than in winter, matching patterns of microbial densities shed by birds, but not those of airborne microbes (Fig. 3). Explanations for seasonal variation in immune function are divergent: some authors predict reduced immunity during a challenging season due to resource trade-offs (e.g., Buehler et al. 2008, Martin et al. 2008); others predict increased immunity during hard times, possibly in anticipation of an increased need for protection (Nelson and Demas 1996, Nelson et al. 2002). These are mostly studies of temperate environments, where winter is often considered the challenging season because short days limit foraging time, food availability is reduced, and thermoregulatory requirements elevate energy demands. Our study contrasts such temperate studies because, in deserts, spring and summer may pose challenges for survival. High temperatures restrict foraging time (Tieleman and Williams 2002), food availability is low, and evaporative cooling requirements increase demands for water (Tieleman et al. 2003*c*), which must primarily be obtained from food, since free-standing water is scarce. Thus, our data support the second prediction: constitutive innate immunity was higher when conditions were more demanding, and when the need for protection, as judged by densities of bird-associated microbes, was greater. Additional investigations are required in order to determine whether heightened immunity during spring is an evolutionary response to a greater need for protection during that season, or a shorter-term physiological response (van de Crommenacker et al. 2010) to higher densities of microbes on birds that equates to higher levels of innate immunity. Further insight into these possibilities might arise from the use of techniques that quantify non-culturable microbes. Viruses, for example, also show patterns of seasonality in abundance and viability (Altizer et al. 2006, Lowen et al. 2007, Tang 2009). Quantification of relevant viruses directly (e.g., via mucosal swabs) and indirectly (e.g., via antibody titers), in conjunction with the assays used here, would broaden substantially our ability to match seasonal patterns of immunological triggers and responses.

Inter-annual variation in constitutive innate immunity

Across all species, regardless of nomadic habits, immune indices were consistently higher in spring 2007 than spring 2006. Body masses were also higher in birds sampled in spring 2007 (Table 2). Larks in the desert skip breeding during lean years, favoring self-maintenance activities over reproduction during such times (Tieleman and Williams 2002, Tieleman et al. 2003*c*, Tieleman et al. 2004). In spring 2007, decreased food availability in Mahazat reduced breeding activities among residents and increased dispersal of nomads (B. I. Tieleman, *personal observation*). Apparently, in this lean year, larks allocated more resources to body mass and immune defenses, perhaps to increase chances of survival until future breeding attempts.

Inter-annual variation was greater than seasonal variation in constitutive innate immune function (Table 2). Similar results have been observed in skylarks (*Alauda arvensis*) living in a mesic environment (A. Hegemann, *unpublished data*). Despite differing in multiple characteristics (e.g., temperature, aridity, and primary productivity), desert and temperate environments both appear to display an inter-annual unpredictability that may be important for shaping the immune function of their inhabitants. These findings emphasize the value of multi-year studies for understanding immunological variation.

Lysis titers correlated with total microbial densities shed from birds

The positive correlation between lysis titer and total microbial load shed by birds (Fig. 6) indicates that also at the individual level, bird-associated microbes may be important for shaping immune function. Lytic mechanisms might be particularly important for dealing with environmental microbes since lysing foreign cells is a critical first step in their neutralization and removal (Janeway et al. 2004). Huttunen et al. (2010) show that in vitro cell cultures release inflammatory cytokines when exposed to suspensions of microbial particles collected from ambient air and that more concentrated suspensions lead to greater release of these immune markers. These results, which are in line with our more ecologically-oriented results, suggest a positive relationship between exposure to environmental microbes and immune responses. Future studies should take advantage of culture-independent techniques, which will further broaden our understanding of host-dependent and host-independent microbial pressures and their relation to immune defenses.

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